

BEST AVAILABLE COPY

EXHIBIT E

Establishment and some characteristics of epoxomicin (a proteasome inhibitor) resistant variants of the human squamous cell carcinoma cell line, A431

KIYOSHI OHKAWA, TADASHI ASAKURA, KATSUHIKO AOKI, SHUN-ICHI SHIBATA, JIRO MINAMI, CHIEKO FUJIWARA, TOMOAKI SAI, HIDEKI MARUSHIMA and HIROFUSA KUZUU

Department of Biochemistry, Jikei University School of Medicine, Tokyo, Japan

Received August 20, 2003; Accepted October 13, 2003

Abstract. A431 resistant variants to epoxomicin (EXM) were established, showing 4.0-6.7 times more resistance to EXM than parental A431P. Both variants demonstrated increased expression of the β -subunit molecules of 26S proteasome with approximately 2.5 times increased activity. In variant cells, cyclin B and P34^{cdc2} were over-expressed, whereas P21^{WAF1} was expressed at a similar level to A431P. Because of the proteasome inhibitor acting as a G2/M blocker, results are to the advantage of resistant cells proliferating in the presence of an inhibitor under a severe environment. Variant cells showed increased expression of epidermal growth factor receptor (EGFR) and decreased expression of mRNA, but also slight accumulation of protein of c-Cbl, which is a negative regulator of EGFR possessing ubiquitin ligase activity to desensitize EGF signaling. Ubch7, acting intimately with c-Cbl, was decreased in level compared to A431P. These phenomena can be regarded as one of the causes of prevention of c-Cbl-mediated down-regulation of EGFR in variant cells, enabling them to live. The anti-apoptotic Bcl-2 mainly consisted of a phosphorylated form with resistance to proteasomal degradation, suggesting that Bcl-2 phosphorylation occurred independently of its apoptotic function. Variant cells showed resistance not only to EXM, but to the 5 proteasome inhibitors, while demonstrating collateral sensitivity to doxorubicin.

Introduction

The ubiquitin/proteasome system is an extralysosomal, ATP-dependent protein degradation system. It participates in the control of many cellular signals that concern proliferation,

growth, differentiation and death, namely apoptosis, of the cells (1-6). It is the 26S proteasome that serves as the center of this degradation mechanism and is within the huge enzyme complex, which acts as the center of the functional control of the cell (1-7). The suppression of proteasome activity that participates in many life phenomena leads to death, i.e. apoptosis of the cell. The use of such inhibitors for this multi-functional enzyme complex, 'proteasome' as anti-cancer drug has been attempted in recent years (8-20). Proteasome inhibitor is a drug with highly anticipated efficacy as an anti-cancer drug for clinical use. To evaluate the possible generation of cells resistant to the inhibitor and their specific properties it is necessary to work out a strategy for the second line chemotherapy. PS-341, an inhibitor, is already undergoing a clinical-phase trial for several malignant tumors including multiple myeloma (9,10,12,17,21,22). However, there are scarce data available on the clinical use of a proteasome inhibitor as an anti-cancer drug. In addition to noting any systemic side effect, if cancer cells acquiring resistance to a proteasome inhibitor reappear after inadequate or incomplete cancer therapy, the very careful use of this type of agent is necessary. Therefore, a squamous cell carcinoma cell line (A431EXM) resistant to epoxomicin (EXM) (23), which is a proteasome-specific inhibitor, was established and some features were examined to overcome the resistance to treatment.

Materials and methods

Materials. EXM, N-benzoyloxycarbonyl-Leu-Leu-Leu-CHO (MG132), N-acetyl-Leu-Leu-Nle-CHO (ALLN), N-benzoyloxycarbonyl-Ile-Glu(OBu)-Ala-Leu-CHO (PSI), and lactacystin were purchased from the Peptide Institute (Osaka, Japan). 4-Hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (NLVS), N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarine (suc-LLVY-AMC), acetyl-Asp-Glu-Val-Asp- α -(4-methyl-coumarinyl-7-amide) (Ac-DEVD-MCA) and doxorubicin (DXR) were obtained from Calbiochem (San Diego, CA, USA). λ -protein phosphatase was purchased from New England Biolabs Inc. (Daiichi Pure Chemicals, Tokyo, Japan). All other chemicals were reagent grade.

Cells and culture. The human squamous cell carcinoma cell line, A431 (designated as A431P in this study), obtained from

Correspondence to: Dr K. Ohkawa, Department of Biochemistry, Jikei University School of Medicine, 3-25-8 Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan
E-mail: pko@jikei.ac.jp

Key words: proteasome inhibitor, epoxomicin resistance, human squamous cell, carcinoma, phosphorylated Bcl-2

the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan), was maintained in Dulbecco's modified minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum (FBS). The human erythrocytic leukemia, multidrug resistant (MDR) variant cell line, K562/DXR, was maintained in RPMI 1640 with 10% FBS and 200 nM DXR as reported previously (24). Cell passage was carried out once weekly.

Establishment of EXM-resistant variants. EXM-resistant variants of A431 were obtained by exposure to EXM as follows. Initial induction of resistance was achieved by continuous exposure of A431 cells to EXM (6.25 nM) over 2 months. Growing resistant cells were further treated with gradually increasing concentrations of EXM (increasing every 4 weeks) until the concentration finally reached 12 nM of EXM. The resistant A431 cells that survived exposure to 12 nM EXM were designated as A431EXM. A431EXM cells were cloned by the limiting dilution method in a 96-well culture plate. After the cytotoxic assay, two clones named A431EXM-1 and A431EXM-2 were maintained in the presence of 12 nM of EXM.

Cytotoxic assay. Harvested cells were washed, seeded and cultured for 17 h without EXM or DXR. Then, cytotoxicity of DXR or various proteasome inhibitors as described in Materials and methods was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay after a 72-h incubation as reported previously (25). Physiologic (0.9%) saline and dimethyl sulfoxide (DMSO) were used as controls, respectively. The results were expressed by the following equation: viable cells (%) = 100 × (absorbance at 570 nm of the treated cells)/(absorbance at 570 nm of the untreated control cells).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Cells were harvested and cell lysates were prepared using lysis buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, 1% Triton X-100, 1 µg/ml aprotinin, leupeptin) as described previously (26). SDS-containing and heat-denatured cell lysates were electrophoresed and the gels were transblotted to nitrocellulose filters. After blocking with bovine serum albumin, the blots were incubated with the corresponding primary antibodies followed by either alkaline phosphatase- or horseradish peroxidase-labeled anti-mouse IgG and the signals were developed with an alkaline phosphatase substrate or enhanced chemiluminescence (Pierce, Rockford, IL, USA). The primary antibodies used were the anti-multi-ubiquitin chain (FK 2, MBL, Nagoya, Japan), -P32 and -P27 of a cylinder particle in the 20S proteasome (Progen Biotechnik, Heidelberg, Germany), -P21^{WAF1}, -cyclin B, -P34^{cdc2} (cyclin-dependent kinase 1, CDK1), -Bcl-2, -UbcH7, -epidermal growth factor receptor (EGFR), -c-Cbl (Transduction Laboratories, Lexington, KY, USA), -P-glycoprotein (P-gp, C219, Centcor, Malvern, PA, USA), and -actin (C-2, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein content of the samples was determined using a Coomassie protein assay (Pierce).

Assay for proteasome activity. Proteasome activity in cytosolic extracts was quantified using the fluorogenic proteasome substrate, suc-LLVY-AMC. Cytosolic extract (50 µg protein in 20 µl) was incubated in a 100-µl reaction mixture containing 50 mM HEPES, pH 7.5, 2 mM dithiothreitol, 0.035% SDS, 10% glycerol, and 100 µM suc-LLVY-AMC at 37°C for 10 min. Fluorescence was determined at 380 nm excitation/460 nm emission, by means of a spectrofluorophotometer (RF-5300PC, Shimadzu, Tokyo, Japan).

Phosphatase treatment. Cells lysates were prepared using 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 1 µg/ml aprotinin, 1.5 mM MnCl₂. Lysates were incubated with or without λ-protein phosphatase (4 units/µl) for 0.5, 1, or 30 min at 30°C. The reaction was terminated by adding SDS-containing sample buffer and boiling for 5 min.

Assay for caspase 3 activity. Caspase 3 activity was determined as described previously (25). Briefly, 100 µg of cell lysates and 100 µM of Ac-DEVD-MCA, substrate, in 50 mM HEPES, pH 7.5, 10% glycerol and 2 mM dithiothreitol, were incubated for 10 min at 37°C. Fluorescence was measured using the method mentioned above.

Expression levels of c-Cbl, EGFR, cyclin B and CDK 1 mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using TRIzol LS reagent (Gibco-BRL, Tokyo Japan) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 µg of RNA using the TrueScript II Reverse Transcriptase kit (Sawady Technology Inc., Tokyo, Japan). Reverse transcription was performed for 10 min at 25°C and 1 h at 55°C, and the samples were subsequently heated for 5 min at 95°C to terminate the RT reaction. With the cDNA obtained, a PCR reaction was performed using 1 µl of the RT reaction mixture as a template. The PCR reaction mixture consisted of 0.5 units of Ex Taq polymerase (Takara, Tokyo, Japan), 200 µM dNTP mixture, 1 µM of sense primer, and 1 µM of antisense primer in 20 µl of the final reaction volume. The tubes were incubated in a GeneAmp PCR system 9700 (Perkin-Elmer, Applied Biosystems, Foster City, CA) at 94°C for 1 min to denature the primers and cDNA. The cycling program was 98°C for 10 sec, 55°C for 30 sec, 72°C for 1 min, and for 10 min in the last cycle. The number of cycles was 30 for all molecules. For the PCR reaction, β-actin was used as the internal control. The following primer sequences in each gene were used: c-Cbl sense 5'-CCCTTGGAAG AGCTTTCGAC-3', c-Cbl antisense 5'-CCCACTGACCA GACGAGTA-3' (311 bp as PCR product); EGFR sense 5'-ATGCGACCCTCCGGGACGGCC-3', EGFR antisense 5'-CCCGGGGGCCTGTGCAGCCTG-3' (733 bp as PCR product); cyclin B sense 5'-TTAATGCTGAAAATAAG GCG-3', cyclin B antisense 5'-CAATTATTCTGCATGA ACCG-3' (680 bp as PCR product); CDK 1 sense 5'-GGT TCCTAGTACTGCAATTCG-3', CDK 1 antisense 5'-TTTGC CAGAAATTCGTTTGG-3' (709 bp as PCR product); and β-actin sense 5'-AACACCCAGCCATGTAC-3', β-actin antisense 5'-ATGTCACGCACGATTTC-3' (254 bp as PCR product).

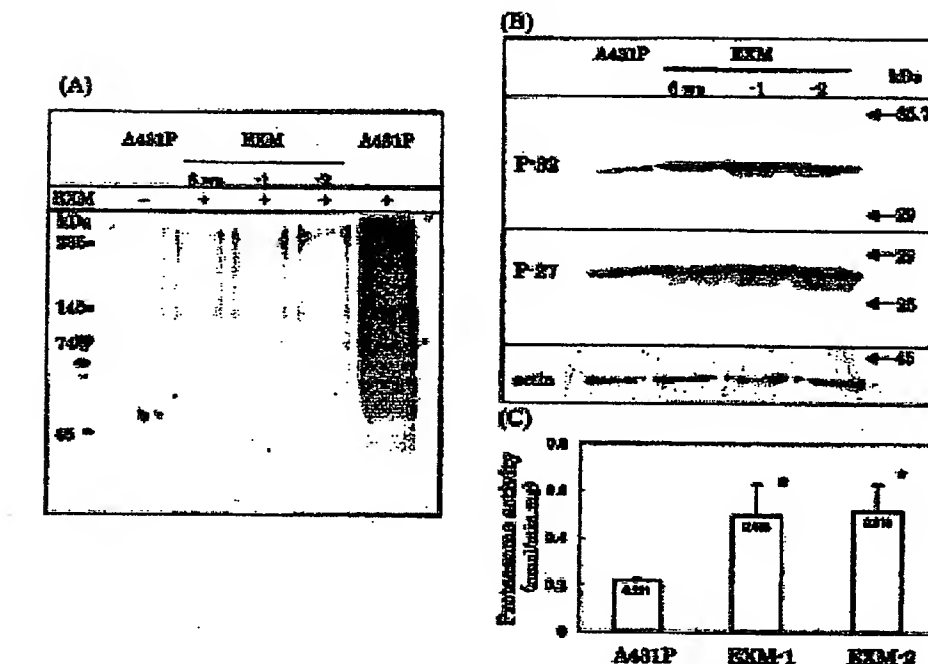


Figure 1. EXM-resistant cell lines show increased proteasome activity. (A), Non-accumulation of multi-ubiquitinated proteins in resistant cells. A431P and EXM-resistant cells (A431EXM-1, -2) before (6ws) and after cloning (-1, -2) were treated with DMSO (-, as control) or 20 nM EXM for 7 h. Cell lysates were prepared by the method described in Materials and methods. After SDS-PAGE/Western blot analyses, resultant filters were probed with FK2 antibody. (B), Increased expression of β -subunits of 20S proteasome. Western blotted filters were probed with anti-P32 and -P27, recognizing β -subunits molecules of cylinder particles in the 20S proteasome. Anti-actin antibody was used as a loading control. (C), Elevated proteasome activity in resistant cells. The measurement of activities in the lysates was carried out using fluorogenic peptide substrate, suc-LLVY-AMC as described in Materials and methods. Results represent the mean \pm SD of duplicate determinations of 2 independent experiments. Statistical significance: * $P < 0.05$, relative to A431P samples based on the Student's t-test. kDa, kilodalton.

Results and Discussion

Establishment of resistant cell lines. Initial induction of resistance was achieved by continuous exposure of A431 parental cells (A431P) to EXM over 3 months, and two stable clones (A431EXM-1; $IC_{50} = 14$ nM, A431EXM-2; $IC_{50} = 20$ nM) showing 4.0-6.7 times more EXM resistance than A431P ($IC_{50} = 3.6$ nM) were established.

Some biochemical features of resistant cell lines. Despite the influence of EXM, neither of the resistant cell lines showed any accumulation of high molecular weight ubiquitinated proteins, which was a typical reaction after the treatment of cells with a proteasome inhibitor (Fig. 1A) (5,27). The qualitative and quantitative change of the proteasome activity of both resistant cell lines was adequately estimated. Actually, the expression of β -subunit molecules, P32 and P27, of the cylinder particle in the 20S proteasome increased in each clone as determined using Western blot analyses (Fig. 1B). It has been reported that in several diseases (28,29), such qualitative and quantitative change of the subunit parts in the 20S proteasome was detectable. It may be useful to further-analyze the proteasomal subunit change in individual diseases in the future. The increased protein levels of subunit molecules were well correlated with the proteasome activity estimated

by means of suc-LLVY-AMC degradation (Fig. 1C). This is the first report for the establishment of resistant cell lines against proteasome inhibitor with constant up-regulation of the 26S proteasome subunits, although transient up-regulation of the enzyme subunits was noted by treatment of cells with the proteasome inhibitor (30).

Some representative molecules involving mainly cell proliferation, those were degraded via a proteasome-dependent system, in the resistant cells were determined (Fig. 2). Non-detectable proteasome inhibition in resistant cells was confirmed from the fact that the protein level of P21^{WAF1}, cyclin-dependent kinase inhibitor, which is degraded by proteasome, was expressed in the resistant cell lines to the same degree as control A431P cells (Fig. 2A). It has been reported that the tumor suppressors, P53, P27, and P21^{WAF1}, are degraded by the proteasome and treatment with proteasome inhibitors in general up-regulates their intracellular concentration (12,30-33). Since the expression of P21^{WAF1} in resistant cells in this study was at a similar level to that of control cells without any obvious accumulation, it can be assumed that the resistant cell lines maintain sufficient ability to proliferate. It has been demonstrated that the A431P cell line lacked activity of the P53 tumor suppressor gene product with the gene mutation (34). It is also known that P21^{WAF1} is a downstream product of the P53 tumor

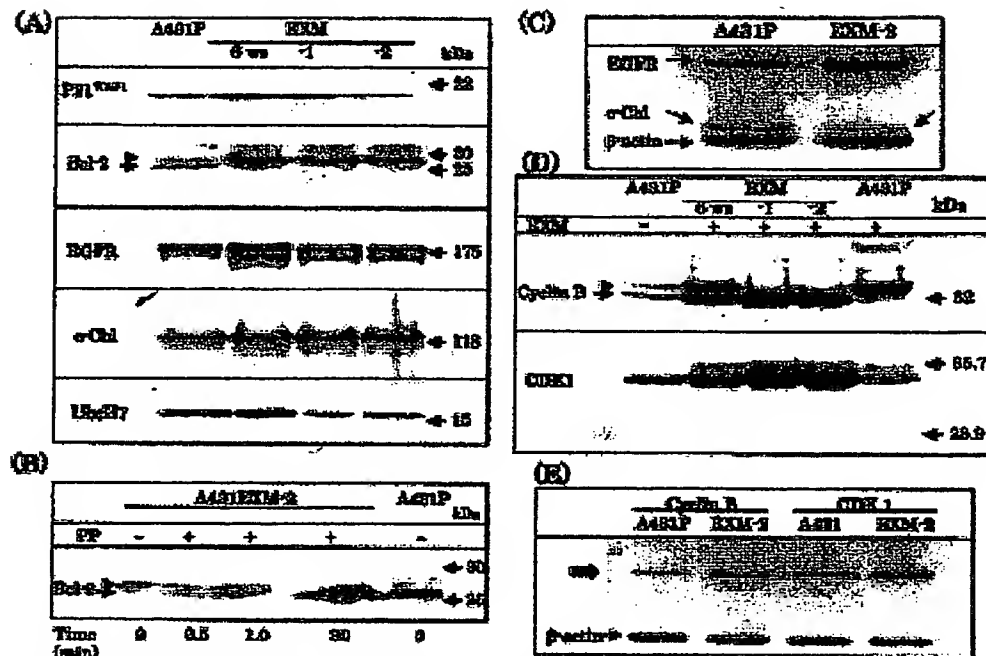


Figure 2. Expression of growth-related proteins in EXM-resistant cell lines. (A), A431P and EXM-resistant cells were cultured with growth media and with growth media supplemented with 14 or 20 nM EXM, respectively. After preparing the cell lysates, SDS-PAGE/Western blot analyses were performed using the respective antibodies. In Bcl-2, the arrowhead indicates the slower migrating Bcl-2 bands. (B), A431EXM-2 cell lysates were incubated with (+) or without (-) λ -protein phosphatase (PP) for 0.5, 1.0, or 30 min at 30°C. SDS-PAGE/Western blot analysis was performed using anti-Bcl-2 antibody. The arrowhead indicates the slower migrating Bcl-2 bands. (C and E), RT-PCR analyses of EGFR, c-Cbl, cyclin B and CDK1 mRNA expression in A431P and EXM-resistant variant cells were determined. Total RNA was reverse transcribed in Materials and methods. β -actin fragment was used as an internal control. The size of the products was determined from the mobility of the DNA size marker. (D), Protein expression of cyclin B and CDK1. Each group of cells was treated as in Fig. 1A. After preparation of the cell lysates, SDS-PAGE/Western blot analyses were carried out. For cyclin B, the 2nd arrowhead indicates the slower migrating, probable ubiquitinated bands. kDa, kilodalton.

suppressor gene, but its accumulation after proteasome inhibition is not always P53-dependent (9). Since a transient but obvious over-expression of P21^{WAF1} in cells continuously exposed to EXM, compared with that in control non-treated A431P cells, was detectable during the process of acquiring resistance to EXM (Fig. 2A, indicated as 6ws), it can be deduced that P53-deficient A431 cells usually express P21^{WAF1}. In EXM-resistant cells, cyclin B and CDK1, both mRNAs and proteins, were over-expressed as compared with those in parental A431P cells. These cellular responses may be necessary to maintain proliferating activity under the G2/M phase blocking effect of EXM (Fig. 2D and E) (9,17). EXM-resistant cells over-expressed the cyclin B band (62 kDa) with faster migration. It is well known that the cell cycle progression requires several checkpoints (35) and progression through the eukaryotic cells requires the activity of a set of distinct cyclin-CDK complexes (36). A complex of CDK1 and cyclin B, mitotic cyclin, which accumulates during interphase to drive the initiation of mitosis and is degraded by the ubiquitin/proteasome system at the end of mitosis to reset the cycle, has been known as the essential factor for G2/M traverse as well as cell cycle progression (37-39). Since degradation of cyclin B is essentially required for the transition from telophase into the subsequent interphase, the

cyclin B mutation with the destruction box drives the cell into mitosis, but arrests cell division in telophase, leading to apoptosis (38-40). In contrast to EXM-resistant cells, transient 7-h treatment of A431P cells with EXM (20 nM) led to accumulation to an obviously higher level of cyclin B with a slower migrating band (64 kDa), which probably was the ubiquitinated form, and which was also detectable but only at a slight level in A431P cells (Fig. 2D, arrowhead). These results were recognized as reasonable intracellular modifications for cellular growth to overcome and adapt to the toxic effect of proteasome inhibitor.

Bcl-2, one of the key factors of apoptosis, in EXM cells was discernible as two bands of different electrophoretic mobility: a small but detectable level estimated to be an approximately 26-kDa faster migrating band corresponding to unmodified Bcl-2, and a main slower migrating 28-kDa band representing the phosphorylated form of the Bcl-2 protein (17,41,42) rather than the unmodified protein (Fig. 2A). The treatment of cell lysates prepared from EXM-resistant cells with λ -protein phosphatase resulted in the time-dependent acceleration of the mobility of slower migrating 28-kDa band, indicating that the Bcl-2 in EXM-resistant cells was mainly phosphorylated (Fig. 2B). The slower migrating band was also detected in the control parental cells, but the expression

was 1/10 less than that of EXM (Fig. 2A, arrowhead). The role of phosphorylation of Bcl-2 in activating or inhibiting its apoptotic function remains controversial. Under the influence of anti-cancer agents such as a microtubule-damaging agent, several reports have shown that they induced Bcl-2 phosphorylation and inhibited its anti-apoptotic function (43-49). However, other reports demonstrated that the Bcl-2 phosphorylation occurred independently of its anti-apoptotic function and the phosphorylated Bcl-2 appeared to be resistant to proteasome-induced degradation (41,50-53) although Bcl-2 is a protein known to be degraded by proteasome (54). This suggests that Bcl-2 phosphorylation could play an indirect, but essential role via protection against degradation although it does not seem to directly regulate the anti-apoptotic function of Bcl-2. In the presence of EXM in the medium, the existence of cells with accumulation of phosphorylated Bcl-2, which is stable against proteasome-dependent proteolysis, is advantageous for protection from apoptosis.

It is well known that the A431P cell over-expresses EGFR, which is a receptor protein tyrosine kinase (34). Both EXM-resistant cell lines expressed higher amounts of EGFR-mRNA and -protein than the control A431P cell line (Fig. 2A and C). EGF when bound to its receptor triggers an allosteric activation of the intracellular tyrosine kinase domain and this activation is essential for the stimulation of various biochemical processes, and signaling pathways leading to cell proliferation (55). EGF-induced EGFR desensitization is accomplished by accelerated endocytosis and degradation of ligand-receptor complexes (56,57). Over-expression or delayed-degradation of the EGFR in the EXM-resistant cells is a quite reasonable and essential cellular response to maintain the dynamic proliferating activity under such severe life-and-death circumstances in the presence of toxic concentrations of proteasome inhibitors. In EXM-resistant cells, it was found simultaneously that c-Cbl-protein, an E3 ubiquitin ligase containing the RING finger domain (58-63) also had increased (Fig. 2A). Ubiquitination of the EGFR is necessary for intracellular trafficking and degradation of the receptor. c-Cbl protein, known to be a negative regulator possessing enzyme activity of E3 for EGFR, directly controls down-regulation of the receptor by recruiting ubiquitin-activating (E1) and -conjugating (E2) enzymes. c-Cbl-mediated receptor sorting involves covalent attachment of ubiquitin molecules, and subsequent lysosomal and proteasomal degradation. In general, a correlation was found between the expression of c-Cbl and the activity of several receptor protein tyrosine kinases. For example, over-expression of c-Cbl protein increased the rate of ligand-induced degradation of EGFR (58,60,62,64,65); conversely, higher EGFR autophosphorylation was observed in cells expressing lower levels of c-Cbl (64,66). In the present study of EXM-resistant cells with over-expression of EGFR, a similar result to that described above was recognized as the message of c-Cbl was extremely reduced as compared to that of parental cells, in spite of the increased protein level of c-Cbl (Fig. 2A and C). This fact suggested that reduction or inhibition of the c-Cbl enzyme activity could enhance recycling and probably accumulation of EGFR on the plasma membrane, without entering the degradation pathway. It is known that c-Cbl protein and Cbl-interacting protein of 85 kDa (CIN85)

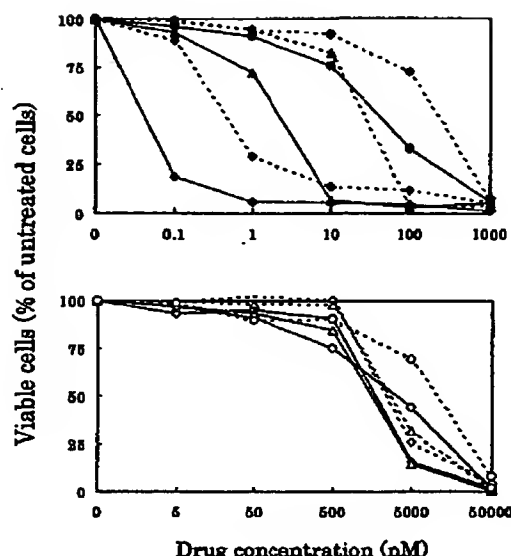


Figure 3. Cytotoxicity of various proteasome inhibitors on the EXM-resistant cell line, A431EXM-2. A431P and the resistant cells (1×10^4) were cultured with the test materials and DMSO as the control. After a 72-h incubation, the cell viability was determined by the MTT assay. Results are the means of triplicate independent experiments. A431P versus PSI (—), EXM (—), MG132 (—), ALLN (—), NLVS (—), lactacystin (—). A431EXM-2 versus PSI (—), EXM (—), MG132 (—), ALLN (—), NLVS (—), lactacystin (—).

(67,68) are usually degraded together with EGFR and the degradation of EGFR is complete, whereas degradation of the CIN85 and c-Cbl proteins is partial in the pathway (69). It is likely under such conditions that c-Cbl acts upstream to the proteasomal and lysosomal degradation processes. In general, most c-Cbl proteins, which are ubiquitinated after ligand-induced stimulation (70), are not degraded but recovered and recycled in a de-ubiquitinated form in the cytoplasm (66,69). This may be one reason why resistant cells accumulated the c-Cbl protein despite the severe reduction of its mRNA. It is well known that the ubiquitin-conjugating enzyme H7 (UbcH7, one of E2 family) is associated intimately with the c-Cbl protein in c-Cbl-mediated ubiquitination and desensitization of the phosphorylated EGFR after ligand-induced activation (60,61,71). In EXM-resistant cells, protein expression of UbcH7 was extremely low as compared with that in parental cells (Fig. 2A), and decreased levels of the UbcH7 must be one of the causes of prevention of c-Cbl-mediated down-regulation of EGFR in resistant cells. The possibility of decreased expression of either E1 or the de-ubiquitinating enzyme cannot be denied because of the low-level accumulation of multi-ubiquitinated proteins with a high molecular weight in EXM-resistant cells. Further examination will be necessary to clarify the precise regulatory mechanism of the ubiquitin/proteasome system in EXM-resistant cells.

Cytotoxic efficacy of various proteasome inhibitors. As shown in Fig. 3, when EXM-resistant cells were treated with 6 proteasome inhibitors, they exhibited higher viability in the

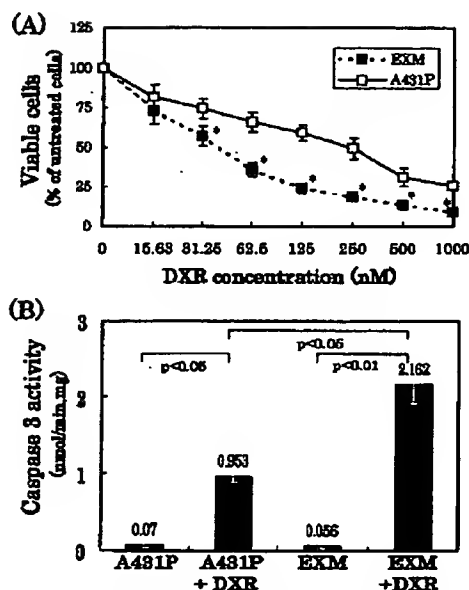


Figure 4. Effect of DXR on cytotoxicity and caspase 3 activity in EXM-resistant cell line, A431EXM-2. (A), EXM-resistant cells showed increased sensitivity of the toxic effect against DXR. A431EXM-2 or A431P cells (1×10^4) were cultured with various concentrations of DXR. After a 72-h incubation, cell viability was determined as Fig. 3. Results represent the mean \pm SD (bar) of duplicate determinations of 2 independent experiments. Bars indicated unless smaller than the points as plotted. * $P < 0.05$, relative to A431P cells (A431P), based on the Student's *t*-test. EXM, A431EXM-2 cell line. (B), Extreme elevation of caspase 3 activity by the treatment of A431EXM-2 cells with DXR. A431P or A431EXM-2 cells were treated with or without 1.5 μ M DXR for 12 h. The measurement of enzyme activities in lysates was carried out using the fluorogenic peptide substrate, Ac-DEVD-MCA as described in Materials and methods. Results represent the mean \pm SD of duplicate determinations of 2 independent experiments. Statistical significance was calculated based on the Student's *t*-test.

presence of all inhibitors than parental cells in spite of various degrees of sensitivity. This finding indicated that the characteristic features of the EXM-resistant cell lines generated in this study were sharing a common and basic mechanism of resistance with proteasome inhibitors.

Doxorubicin (DXR) sensitivity of the resistant cells. As shown in Fig. 4A, the increased DXR-sensitivity in an EXM-2-resistant cell line was demonstrated and the IC_{50} of DXR on the resistant cell line (40 nM) was about 5.8-fold lower than that on A431P cells (230 nM). Supporting this finding, caspase 3 activity in the A431P cells increased more than 13.6-fold after 12 h of incubation with 1.5 μ M DXR (IC_{50}) as compared to the untreated parental cells. In contrast to parental cells, the resistant cells unexpectedly showed an elevated caspase 3 activity up to a 38.6-fold higher level as compared to the untreated resistant control, in response to the same treatment. Consequently, the EXM-resistant cell line was approximately 2.38-fold more sensitive to DXR than parental cells, indicating a positive result of the DXR-sensitivity test (Fig. 4B). Taken together, these findings suggest that the resistant cell line against proteasome inhibitors may

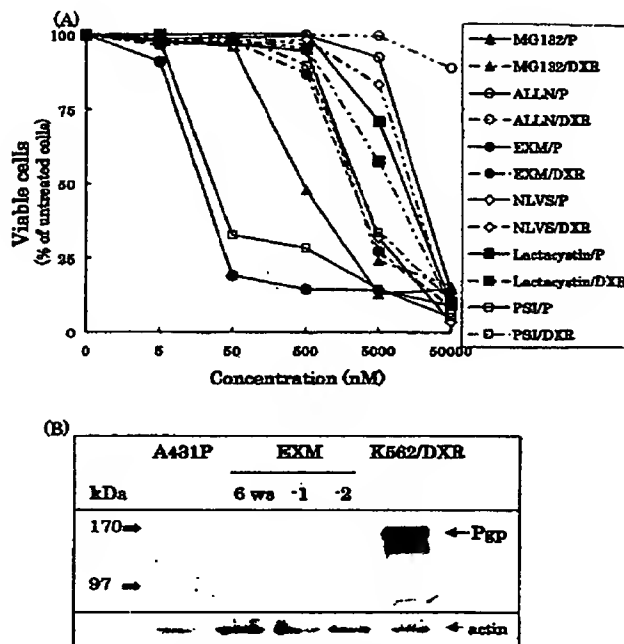


Figure 5. P-gp protein causes a reduced cytotoxic efficacy of proteasome inhibitors. (A), Reduced cytotoxicity of proteasome inhibitors against K562/DXR cells. K562/DXR and parental K562 cells (1×10^4) were cultured with various proteasome inhibitors at graded concentrations. Viable cells were determined by the MTT assay after 72 h of incubation. DMSO as a control. Results represent the means of triplicate experiments. In the legend, MG132/P means K562 parental cells versus MG132 and MG132/DXR means K562/DXR cells versus MG132, and so on. (B), A431P, A431EXM-1, -2, pre-cloned EXM-resistant (6ws) cells and multi-drug resistant, P-gp over-expressing, K562/DXR cells were cultured and cell lysates were prepared. SDS-PAGE/Western blot analysis using C219 antibody was performed. Anti-actin antibody was used as a loading control. kDa, kilodalton.

have possessed more apoptosis-inducible, sensitive features in response to certain kinds of anti-cancer agent. The anti-cancer drugs, DXR, VP-16 and VM-26, are classified as DNA-topoisomerase (Top) II inhibitors and the anti-tumor activity of these agents is attributable to their specific interference with the breakage/reunion reaction of DNA-TopII (72-76). These drugs result in the accumulation of a reversible covalent reaction intermediate, which is the so-called cleavable complex. It has been demonstrated that a possible new mode of action of this category of anti-cancer drug (77) based on the fact that proteasome inhibitors can block VP-16 or VM-26 induced apoptosis without any inhibition of NF- κ B activation. The stabilized cleavable complexes induced by this type of drug can trigger ubiquitin conjugation to TopII resulting in 26S proteasome-mediated degradation of TopII (77). Moreover, the TopII cleavable complex is degraded via the ubiquitin/proteasome system (77). Proteolysis of TopII cleavable complexes with the ubiquitin/proteasome system results in exposure of the protein-concealed double strand breaks. The exposed double strand breaks can then undergo repair; to the contrary un-repaired double strands can trigger apoptotic cell death. Down-regulation of TopII must be a

critical event for DNA repair or unrepair. The involvement of the ubiquitin/proteasome system, which is also recognized in TopI-regulation with camptothecin (CPT-11) treatment (74,76), must have a significant influence of the efficacy on the Top-targeting anti-cancer drug. The present result suggests that EXM-resistant cells with an increased level of 26S proteasomal activity showed superior cytotoxic sensitivity to DXR relative to their parental cells. This result also suggests that second line chemotherapy using a TopII inhibitor such as DXR and VP-16 (etoposide) or TopI inhibitor (CPT-11) should be conducted although the treatment of cancer patients with proteasome inhibitors may generate this kind of tumor cell with a proteasome inhibitor-resistance phenotype. This theory may explain, although further study will be necessary, why several studies demonstrated superior cytotoxic activity in combination with CPT-11 and PS-341 (78,79).

Resistance to proteasome inhibitors is linked to P-gp expression. Five out of 6 proteasome inhibitors including EXM, used in this study to generate the cells resistant to proteasome inhibitor, were actively effluxed from the MDR cell line, K562/DXR, because of the reduced toxic efficacy of the inhibitors (Fig. 5A). This indicated that these 5 inhibitors, except for lactacystin, might act as a substrate, like ALLN (80,81), for P-gp, a member of the ATP-binding cassette transporters with a 170-kDa integral membrane protein encoded by the MDR gene. As shown in Fig. 5B, detectable levels of P-gp molecules, which are usually over-expressed in MDR cells (80,81), were not found in EXM-resistant A431 cells, suggesting that P-gp was not involved in the acquisition of resistance to EXM. It has been reported that PS-341, a potent proteasome inhibitor currently undergoing evaluation in clinical trials, is a poor substrate for P-gp and other MDR related proteins (10,12). We could not obtain and test PS-341 in our study, however, most of the proteasome inhibitors tested were effluxed from the cells by P-gp, suggesting that careful therapeutic applications of such drugs are necessary in clinical use.

The A431EXM cell line is a reliable tool for therapeutic evaluation with a proteasome inhibitor in pre-clinical trials. Moreover, in order to clarify the mechanism(s) of resistance to the proteasome inhibitor of A431EXM a wide variety of proteasomal functions should be examined and this cell line may also be a useful tool. Many additional experiments are necessary to characterize and establish the cell line.

References

- Varshavsky A: The ubiquitin. *Trends Biochem Sci* 22: 383-387, 1997.
- Ciechanover A: The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J* 17: 7151-7160, 1998.
- Hershko A and Ciechanover A: The ubiquitin system. *Annu Rev Biochem* 67: 425-479, 1998.
- Voges D, Zwickl P and Baumeister W: The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* 68: 1015-1068, 1999.
- Hershko A, Ciechanover A and Varshavsky A: The ubiquitin system. *Nat Med* 6: 1073-1081, 2000.
- Ciechanover A and Schwartz AL: Ubiquitin-mediated degradation of cellular proteins in health and disease. *Hepatology* 35: 3-6, 2002.
- Naujokat C and Hoffmann S: Role and function of the 26S proteasome in proliferation and apoptosis. *Lab Invest* 82: 965-980, 2002.
- Lee DH and Goldberger AL: Proteasome inhibitors: valuable new tools for cell biologists. *Cell Biol* 8: 397-403, 1998.
- Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Mass J, Pien CS, Prakash S and Elliott PJ: Proteasome inhibitors: a novel class of potent and effective anti-tumor agents. *Cancer Res* 59: 2615-2622, 1999.
- Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J and Anderson KC: The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 61: 3071-3076, 2001.
- Shimul AS, Potter MW and Callery MP: Ubiquitin proteasome inhibition and cancer therapy. *Surgery* 131: 595-600, 2001.
- Adams J: Preclinical and clinical evaluation of proteasome inhibitor PS-341 for the treatment of cancer. *Curr Opin Chem Biol* 6: 493-500, 2002.
- Adams J: Proteasome inhibitors as new anticancer drugs. *Curr Opin Oncol* 14: 628-634, 2002.
- Almond JB and Cohen GM: The proteasome: a novel target for cancer chemotherapy. *Leukemia* 16: 433-443, 2002.
- Garber K: On the eve of protein destruction: ubiquitin research begins to pay off. *J Natl Cancer Inst* 94: 550-552, 2002.
- Hernandez AA and Roush WR: Recent advances in the synthesis, design and selection of cysteine protease inhibitors. *Curr Opin Chem Biol* 6: 459-465, 2002.
- Ling Y-H, Liebes L, Ng B, Buckley M, Elliott PJ, Adams J, Jiang J-D, Muggia FM and Perez-Soler R: PS-431, a novel proteasome inhibitor, induces Bcl-2 phosphorylation and cleavage in association with G2-M phase arrest and apoptosis. *Mol Cancer Ther* 1: 841-849, 2002.
- Sakamoto KM: Ubiquitin-dependent proteolysis: its role in human diseases and the design of therapeutic strategies. *Mol Genet Metab* 77: 44-56, 2002.
- Smith DM, Wang Z, Kazi A, Li L-H, Chan T-H and Dou QP: Synthetic analogs of green tea polyphenols as proteasome inhibitors. *Mol Med* 8: 382-392, 2002.
- Yu R, Ren S-G and Melmed S: Proteasome inhibitors induce apoptosis in growth hormone- and prolactin-secreting rat pituitary tumor cells. *J Endocrinol* 174: 379-386, 2002.
- Le Blance R, Catley LP, Hideshima T, Lentzsch S, Mitsiades CS, Mitsiades N, Neubergh D, Goloubeva O, Pien CS, Adams J, Gupta D, Richardson PG, Munshi NC and Anderson KC: Proteasome inhibitor PS-341 inhibits human myeloma cell growth *in vivo* and prolongs survival in a murine model. *Cancer Res* 62: 4996-5000, 2002.
- Orlowski RZ, Stinchcombe TE, Mitchell BS, Shea TC, Baldwin AS, Stahl S, Adams J, Esseltine D-L, Elliott PJ, Pien CS, Gueriolini R, Anderson JK, Depcik-Smith ND, Bhagat R, Lehman MJ, Novick SC, O'Connor OA and Soignet SL: Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. *J Clin Oncol* 20: 4420-4427, 2002.
- Meng L, Mohan R, Kwok BHB, Eloffson M, Sin N and Crews CM: Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity. *Proc Natl Acad Sci USA* 96: 10403-10408, 1999.
- Hatano T, Ohkawa K and Matsuda M: Cytotoxic effect of the protein-doxorubicin conjugates on the multidrug-resistant human myelogenous leukemia cell line, K562, *in vitro*. *Tumour Biol* 14: 288-294, 1993.
- Asakura T, Ohkawa K, Takahashi N, Takada K, Inoue T and Yokoyama S: Glutathione-doxorubicin conjugate expresses potent cytotoxicity by suppression of glutathione S-transferase activity: comparison between doxorubicin-sensitive and -resistant rat hepatoma cell. *Br J Cancer* 76: 1333-1337, 1997.
- Ohkawa K, Tsukada Y, Murae M, Kimura E, Takada K, Abe T and Terashima Y: Serum levels and biochemical characteristics of human ovarian carcinoma-associated antigen defined by murine monoclonal antibody, CF511. *Br J Cancer* 60: 953-960, 1989.
- Ishibashi Y, Takada K, Joh K, Ohkawa K, Aoki T and Matsuda M: Ubiquitin immunoreactivity in human malignant tumours. *Br J Cancer* 63: 320-322, 1991.
- Kumarori A, Tanaka K, Imamura N, Sone S, Orura T, Matsumoto T, Tachikawa T, Shin S and Ichihara A: Abnormally high expression of proteasomes in human leukemic cells. *Proc Natl Acad Sci USA* 87: 7071-7075, 1998.

29. McNaught KSTP, Belizaire R, Jenner PC, Olanow W and Isacson O: Selective loss of 20S proteasome α -subunits in the substantia nigra pars compacta in Parkinson's disease. *Neurosci Lett* 326: 155-158, 2002.
30. Mitsiades N, Mitsiades CS, Paulaki V, Chauhan D, Fanourakis G, Gu X, Bailey C, Joseph M, Libermann T, Treon SP, Munshi NC, Richardson PG, Hideshima T and Anderson KC: Molecular sequelae of proteasome inhibition in human multiple myeloma cells. *Proc Natl Acad Sci USA* 99: 14374-14379, 2002.
31. Dietrich C, Bartsch T, Schanz F, Oesch F and Wieser RJ: p53-dependent cell cycle arrest induced by N-acetyl-L-leucyl-L-leucyl-L-norleucinal in platelet-derived growth factor-stimulated human fibroblasts. *Proc Natl Acad Sci USA* 93: 10815-10819, 1996.
32. Lopes UG, Erhardt P, Yao R and Cooper GM: p53-dependent induction of apoptosis by proteasome inhibitors. *J Biol Chem* 272: 12893-12896, 1997.
33. Sheaff RJ, Singer JD, Swanger J, Smitherman M, Roberts JM and Clurman BE: Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. *Mol Cell* 5: 403-410, 2000.
34. Park DJ, Nakamura H, Chumakov AM, Said JW, Miller CW, Chen DL and Koeffler HP: Transactivational and DNA binding abilities of endogenous P53 in P53 mutant cell lines. *Oncogene* 9: 1899-1906, 1994.
35. Hartwell L: Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* 71: 543-546, 1992.
36. Evans T, Rosenthal ET, Youghblom J, Distel D and Hunt T: Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 33: 389-396, 1983.
37. Minshull J, Blow JJ and Hunt T: Translation of cyclin mRNA is necessary for extract of activated *Xenopus* eggs to enter mitosis. *Cell* 56: 947-956, 1989.
38. Murray AW, Solomon MJ and Kirschner MW: The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* 339: 280-286, 1989.
39. King RW, Deshaies RJ, Peters J-M and Kirschner MW: How proteolysis drives the cell cycle. *Science* 274: 1652-1659, 1996.
40. Murray AW and Kirschner MW: Cyclin synthesis drives the early embryonic cell cycle. *Nature* 339: 275-280, 1989.
41. Brichesse L, Barboile N, Heliez C and Valette A: Bcl-2 phosphorylation and proteasome-dependent degradation induced by paclitaxel treatment: consequences on sensitivity of isolated mitochondria to bid. *Exp Cell Res* 278: 101-111, 2002.
42. Vantighem A, Xu Y, Assefa Z, Piettes J, Vandenheede JR, Merlevede W, De Witte PAM and Agostinis P: Phosphorylation of Bcl-2 in G2/M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal and delays the onset of apoptosis. *J Biol Chem* 277: 37718-37731, 2002.
43. Haldar S, Jeon N and Croce CM: Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* 92: 4507-4511, 1995.
44. Ling Y-H, Tornos C and Perez-Soler R: Phosphorylation of Bcl-2 is a marker of M phase events and not a determinant of apoptosis. *J Biol Chem* 273: 18984-18991, 1998.
45. Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD and Pietersen JA: Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and taxol-induced growth arrest. *J Biol Chem* 273: 30777-30784, 1998.
46. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-Chung YS and Longo DL: Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. *Mol Cell Biol* 18: 3509-3517, 1998.
47. Srivastava RK, Mi Q-S, Hardwick M and Longo DL: Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc Natl Acad Sci USA* 96: 3775-3780, 1999.
48. Yamamoto K, Ichijo H and Korsmeyer SJ: Bcl-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G2/M. *Mol Cell Biol* 19: 8469-8478, 1999.
49. Mai H, May WS, Gao F, Jin Z and Deng X: A functional role for nicotine in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem* 278: 1886-1891, 2003.
50. Ito T, Deng X, Carr B and May WS: Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* 272: 11671-11673, 1997.
51. Ruvolo PP, Deng X, Carr B and May WS: A functional role for mitochondria protein kinase C α in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem* 273: 25436-25442, 1998.
52. Poompanit PB, Chen B and Oltvai ZN: Interleukin-3 induces the phosphorylation of a distinct fraction of Bcl-2. *J Biol Chem* 274: 1033-1039, 1999.
53. Figueroa-Masot XA, Hetman M, Higgins MJ, Kokot N and Xia Z: Taxol induces apoptosis in cortical neurons by a mechanism independent of Bcl-2 phosphorylation. *J Neurosci* 21: 4657-4667, 2001.
54. Dimmeyer S, Breitschopf K and Haendeler J: Diphosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosis and the proteasome pathway. *J Exp Med* 189: 1815-1822, 1999.
55. Vieira AV, Lamaze C and Schmid SL: Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* 274: 2086-2088, 1996.
56. Sorkin A and Waters CM: Endocytosis of growth factor receptors. *Bioessays* 15: 375-382, 1993.
57. Schmid SL: Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Cell Biol* 66: 511-548, 1997.
58. Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Landgon WY, Geginot L, Geiger B and Yarden Y: c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* 12: 3663-3674, 1998.
59. Joazeir CAP, Wing SS, Huang H-K, Levenson JD, Hunter T and Liu Y-C: The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* 286: 309-312, 1999.
60. Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsyanov AY, Alroy I, Iwai K, Reiss Y, Ciechanover A, Lipkowitz S and Yarden Y: Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* 4: 1029-1040, 1999.
61. Yokouchi M, Kondo T, Houghton A, Bartkiewicz M, Horne WC, Zhang H, Yoshimura A and Baron R: Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J Biol Chem* 274: 31707-31712, 1999.
62. Waterman H, Levkowitz G, Alroy I and Yarden Y: The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J Biol Chem* 274: 22151-22154, 1999.
63. Thien CBF, Walker F and Langdon WY: RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. *Mol Cell* 7: 355-365, 2001.
64. Ueno H, Sasaki K, Miyagawa K, Honda H, Mitani K, Yazaki Y and Hirai H: Antisense repression of proto-oncogene c-Cbl enhance activation of the JAK-STAT pathway but not the ras pathway in epidermal growth factor receptor signaling. *J Biol Chem* 272: 8739-8743, 1997.
65. Miyake S, Luper ML Jr, Druker B and Band H: Thetyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor α . *Proc Natl Acad Sci USA* 95: 7927-7932, 1998.
66. Ettenberg SA, Magnifico A, Cuervo M, Nau MM, Rubinstein YR, Yarden Y, Weissman AM and Lipkowitz S: Cbl-b-dependent coordinated degradation of the epidermal growth factor receptor signaling complex. *J Biol Chem* 276: 27677-27684, 2001.
67. Take H, Watanabe S, Takeda K and Yu Z-X: Cloning and characterization of a novel adaptor protein, CIN85, that interacts with c-Cbl. *Biochem Biophys Res Commun* 268: 321-328, 2000.
68. Soubeyran P, Kowanetz K, Szymkiewicz I, Langdon WY and Dikic I: Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* 416: 183-187, 2002.
69. Haglund K, Shimokawa N, Szymkiewicz I and Dikic I: Cbl-directed monoubiquitination of CIN85 is involved in regulation of ligand-induced degradation EGF receptors. *Proc Natl Acad Sci USA* 99: 12191-12196, 2002.
70. Wang Y, Yeung Y-G, Langdon WY and Stanley ER: c-Cbl is transiently tyrosine-phosphorylated, ubiquitinated, and membrane-targeted following CSF-1 stimulation of macrophages. *J Biol Chem* 271: 17-20, 1996.
71. Yokouchi M, Kondo T, Sanjay A, Houghton A, Yoshimura A, Komiyama S, Zhang H and Baron R: Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. *J Biol Chem* 276: 35185-35193, 2001.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.